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METABOLIC COMPONENTS OF THE MAINTENANCE ENERGY EXPENDITURE OF MAMMALIAN SKELETAL MUSCLE

by

Valeta Anne Gregg

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled METABOLIC COMPONENTS OF THE MAINTENANCE ENERGY EXPENDITURE OF MAMMALIAN SKELETAL MUSCLE submitted by Valeta Anne Gregg in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in ANIMAL BIOCHEMISTRY.

Abstract

The intent of this study was to gain a greater quantitative understanding of the costs of Na^+,K^+-ATPase activity and protein synthesis as components of the maintenance or background energy expenditure of mammalian muscle.

The rate of O_2 consumption and its inhibition by ouabain were measured for intact and sliced mouse soleus and diaphragm muscles incubated in vitro in an O_2 electrode system. Slicing lowered ($p<0.05$) the rate of O_2 consumption of soleus and diaphragm muscles but did not cause a significant difference in the extent of ouabain inhibition of respiration. Ouabain caused a 19.7% inhibition of soleus muscle incubated in 1 mM $MgCl_2$ buffer. The response of respiration to ouabain was abolished upon incubation in buffer containing 10 mM $MgCl_2$. Soleus muscle from mice that had been held at an ambient temperature of 5°C for 3 weeks had a greater ($p<0.001$) rate of O_2 consumption than did the soleus muscle of mice held at 24°C. Increased Na^+,K^+-ATPase activity accounted for 20% of the cold-induced muscle thermogenesis.

An in vitro preparation of sternomandibularis muscle from sheep and cattle in which O_2 availability and membrane potential were maintained was developed. O_2 consumption and inhibition of respiration by ouabain were measured for sheep exposed to a warm (25°C) or cold (1°C) ambient temperature for 5 weeks and fed either at maintenance or at the same

level of intake. Cold exposed sheep had a whole body O_2 consumption 24 or 42% greater ($p<0.05$) when fed at the same level as warm exposed sheep, or at maintenance (1400 g alfalfa pellets/d), respectively. Muscle from cold exposed sheep fed at either level of intake exhibited an O_2 consumption rate 48% greater ($p<0.001$) than that of warm exposed sheep. Ouabain (10^{-6}M) inhibited the muscle O_2 consumption of cold exposed sheep by 41.0 to 45.0%, and of warm exposed sheep by 29.0 to 38.0%. Increased energy expenditure at the level of the Na^+,K^+-ATPase accounted for 50 to 80% of the cold-induced muscle thermogenesis.

Ouabain (10^{-5}M) caused an average of 40% inhibition of sternomandibularis muscle respiration for dairy calves aged 10-21d and 7 months, and 7 month old calves from a double-muscled (DM) population of normal (control DM) and heavily muscled (extreme DM) phenotypes. Rate of O_2 consumption was greatest ($p<0.001$) for muscle from 10-21d dairy calves and lowest ($p<0.05$) for control DM calves. The energy expenditure estimated to be required for peptide bond synthesis accounted for 2.0 to 3.3% of the O_2 consumption of the muscle preparations. Rate of tyrosine release, considered to be an indicator of protein degradation, was greatest ($p<0.05$) for muscle from control and extreme DM calves; both dairy groups had similarly low rates of muscle tyrosine release.

Muscle O_2 consumption was greater ($p<0.001$) for lambs at 2 weeks than at 7 weeks of age, and for ewes when

lactating than when dry. Ouabain (10^{-5}M) inhibited muscle O_2 consumption by an average 39% for all animals. Increased energy expenditure at the level of the Na^+,K^+-ATPase accounted for 40% of the increased O_2 consumption rate of muscle from lambs at 2 weeks as contrasted to 7 weeks of age, and 60% of the increased O_2 consumption rate of muscle due to lactation.

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Introduction

The concept of a constant maintenance requirement based on body weight has been a fundamental element of energy nutrition. This concept of a maintenance component of energy expenditure inevitably influences estimates of energetic efficiency in intact animals because the presumed maintenance energy requirement is first subtracted from energy intake and the difference is then used as the input component in assessment of energetic efficiency of production. In contrast to nutritional concepts associated with production, maintenance has not been identified in terms of metabolic events and it has certainly not been established that the metabolic events accounting for maintenance energy expenditures are constant in relation to body weight. The purpose of this study was to identify metabolic components of maintenance or background energy expenditure.

Metabolic processes which could intuitively be suggested to be part of the maintenance energy requirement include the continual background expenditures on active Na^+-K^+ transport, catalyzed by the plasma membrane Na^+,K^+-ATPase, and on the protein synthesis that must occur during protein turnover in animals. It is not possible to measure Na^+,K^+-ATPase activity in vivo without disruption or destruction of supporting vital functions, therefore, efforts were focused on obtaining in vitro measurements of

Na^+,K^+-ATPase activity which would be indicative of the physiological role of the enzyme as a component of the energy expenditure of skeletal muscle. This involved development of an in vitro preparation in which membrane potential and O_2 availability to the sites of respiration were maintained. This preparation was then amenable to measurement of the proportion of respiration required to support active transport of Na^+-K^+ using ouabain as a specific inhibitor and to support protein synthesis as measured by the rate of incorporation of ^{14}C-phenylalanine, and could be used in the examination of the effects of physiological state on these parameters.

I. INHIBITION BY OUABAIN OF THE O_2 CONSUMPTION OF MOUSE (MUS MUSCULUS) SOLEUS AND DIAPHRAGM MUSCLES[1]

A. Abstract

The rate of O_2 consumption of intact, or sliced soleus and diaphragm muscles was measured polarographically with lactate or glucose as the added substrate; the dimensions of the muscles were such that O_2 diffusion should not have limited respiration.

Ouabain (10^{-3}M) inhibited the respiration of intact soleus and diaphragm muscles by 22 and 33% indicating very real importance of $Na^+ + K^+$ transport in the energy metabolism of these muscles. Slicing lowered the rate of O_2 uptake of soleus and diaphragm by 9 and 14%. Ouabain inhibition of respiration tended to be greater for sliced than for intact muscles but the effect of this method of preparation of tissue was not statistically ($p>0.05$) significant.

B. Introduction

It is generally recognized that ATP production in intact coupled cells is regulated by ATP utilization (Ismail-Beigi, 1977); the availability of ADP and, consequently, the rate of free energy expenditure controls the rate of respiration (Chance & Williams, 1956). Active

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[1]A slightly modified version of this chapter has been published. Gregg, V. A. & Milligan, L. P. (1980) Gen. Pharmac. 11, 323.

ion transport, particularly that catalyzed by the Na^+,K^+-ATPase of the plasma membrane, has been thought by some investigators to result in a significant portion of total ADP generation (Racker, 1976), accounting for 20-45% of the energy expenditure of resting cells (Whittam, 1964; Whittam & Blond, 1964). Ouabain, a specific inhibitor of Na^+,K^+-ATPase has been used in attempts to measure Na^+,K^+-ATPase-dependent respiration in coupled systems (Ismail-Beigi & Edelman, 1970). However, ensuing results on the importance of $Na^+ + K^+$ transport as a component of energy expenditure have been contradictory. Work conducted with quarter-sections of rat diaphragm muscle indicated that $Na^+ + K^+$ transport accounts for 40% of total cellular energy expenditures (Asano et al. 1976), while workers using perfused rat liver (Folke & Sestoft, 1977), isolated rat soleus muscle and adipose tissue (Chinet et al. 1977), have concluded that $Na^+ + K^+$ transport accounts for no more than 6% of the energy expenditure of these tissues.

A study was undertaken to measure the Na^+,K^+-ATPase-dependent respiration of intact mouse (Mus musculus) diaphragm and soleus muscles under conditions in which O_2 availability would not limit respiration. A second study was conducted to assess the response of these muscles to ouabain after the tissue was sliced.

C. Experimental

Adult male mice, in the weight range of 24-41 g, were stunned by a blow to the head and bled from the neck. the intact soleus muscles were removed with care to minimize tissue damage following the procedure of Kohn and Clausen (1971). The diaphragm was dissected from the rib attachments and cut through the central connective tissue. This method produces few damaged muscle fibers relative to the number of intact fibers.

Measurement of ouabain-sensitive respiration of whole soleus and diaphragm muscles

The modified Krebs-Ringer bicarbonate buffer used as the incubation medium contained (mM): NaCl, 116.8; KCl, 5.9; $NaHCO_3$, 25.0; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $CaCl_2$, 1.0; lactate, 1.0; pH 7.4. The tissue preparations were incubated individually in 6 ml buffer at 37°C for 1 h without (control) and with 10^{-3}M ouabain. Incubation flask contents were quantitatively transferred to the O_2 electrode chamber and O_2 consumption was then measured with a YSI O_2 electrode. O_2 consumption values were obtained for the combined soleus muscles from two mice; values for the diaphragms of the two mice were obtained separately and averaged. The O_2 consumption of representative preparations was measured initially and at approximately 10 min intervals throughout the incubation period and was found to remain constant indicating adequacy of the experimental conditions

in maintaining tissue metabolism.

Comparison of ouabain-sensitive respiration of whole and sliced muscles

The Na^+-Ringer's buffer used as the incubation medium in this part of the study contained (mM): NaCl, 130.0; KCl, 5.0; NaH_2PO_4, 5.0; $CaCl_2$, 1.0; glucose, 10.0; pH7.4. O_2 consumption values were obtained for whole and sliced muscle following the procedure used for the measurement of the ouabain-sensitive respiration of whole muscles. Soleus muscles were longitudinally sliced by hand using a razor blade into two or three sections; hemidiaphragm muscles were sliced into five or six sections.

Statistical analysis

Results obtained for the ouabain-sensitive respiration of whole soleus and diaphragm muscle were analysed using a three-way analysis of variance with groups as a random source and tissue and incubation as fixed sources of variation. The comparisons of ouabain-sensitive respiration of whole and sliced soleus and diaphragm muscles, as well as that of whole muscles in two buffers, were analysed using four-way analysis of variance with treatment, tissue and inhibitor as fixed sources and groups nested within treatment a random source.

D. Results and Discussion

Although a variety of buffers have been used by previous investigators (Asano et al. 1976; Folke & Sestoft, 1977), an analysis of the values obtained in this experiment for respiration rates and inhibition of respiration by ouabain of whole soleus and diaphragm muscles showed that determinations conducted in Krebs-Ringer bicarbonate buffer with lactate as substrate were not significantly different from those conducted with Na^+-Ringers's buffer with glucose as substrate. The respiration rates measured for whole diaphragm muscle, 4.3 μl O_2/mg tissue dry wt/h in Krebs-Ringer bicarbonate buffer (Table I.1) and 4.5 μl O_2/mg tissue dry wt/h in Na^+-Ringer's buffer (Table I.2), were lower than the reports of 7.7 (Ismail-Beigi & Edelman, 1970) and 7.5 μl O_2/mg tissue dry wt/h (Asano et al. 1976) for quarter sections of rat diaphragm muscle, but the mouse preparations likely include proportionally more connective tissue. The respiration rates measured for whole soleus muscle, 3.7 μl O_2/mg tissue dry wt/h in Krebs-Ringer bicarbonate buffer (Table I.1) and 3.5 μl O_2/mg tissue dry wt/h in Na^+-Ringer's buffer (Table I.2) were slightly less than those measured for whole diaphragm muscle.

Inhibition of respiration of whole soleus and diaphragm muscles by ouabain was greater, but not significantly ($p>0.05$) so in Krebs-Ringer bicarbonate buffer (21.6 and 32.6%)(Table I.1) than in Na^+-Ringer's buffer (14.3 and 15.6%)(Table I.2). The values determined in this study are

consistent with the 15.6-41.3% inhibition reported previously for rat diaphragm muscle (Ismail-Beigi & Edelman, 1970; Asano et al. 1976). The values for ouabain inhibition of respiration for whole soleus muscle in Krebs-Ringer buffer (Table I.1) were significantly less ($p<0.05$) than those of whole diaphragm muscles; this difference was not observed for the values determined in Na^+-Ringer's buffer (Table I.2).

The results of a comparison of respiration rates and of inhibition of respiration by ouabain for whole and sliced tissue are shown in Table I.2. Slicing lowered ($p<0.05$) O_2 consumption of soleus muscle from 3.5 to 3.2 μl O_2/mg tissue dry wt/h and of diaphragm muscle from 4.5 to 3.8 μl O_2/mg tissue dry wt/h (Table I.2). The inhibition of respiration by ouabain, while greater, was not significantly ($p>0.05$) different from that observed in whole tissue; inhibition was 21.9 to 14.3% in sliced and whole soleus muscle and 26.3 and 15.6% in sliced and whole diaphragm muscle (Table I.2).

The extent to which Na^+,K^+-ATPase acts as a pacemaker of energy metabolism in intact, coupled cells is determined by the magnitude of energy that must be expended to maintain intracellular ionic concentrations and counteract the tendency for external Na^+ and internal K^+ to be moved or to leak across the cell membrane in the direction of their diffusion gradients. Changes in the leakage rates would influence this expenditure. The ouabain-sensitive respiration values measured in this study for mouse soleus

and diaphragm muscles certainly support the importance of the role played by $Na^+ + K^+$ transport in the energy expenditure of these tissues.

Chinet et al. (1977) suggested that the large ouabain-sensitive respiration values measured by others for cut or sliced muscle preparations were due to the stimulation of $Na^+ + K^+$ transport above its basal level by the leakage of Na^+ into the cytoplasm of the cut fibers. Clearly, our results do not support this argument. However, it is likely that the low ouabain-sensitive respiration values measured for whole organ preparations (Chinet et al. 1977; Folke & Sestoft, 1977) were obtained under conditions in which O_2 availability limited respiration. The microcalorimetric studies of heat production by whole rat soleus muscle (Chinet et al. 1977) would have entailed a tissue thickness of 1.5-2.0 mm which exceeds the approximate 0.4 mm at which the rate of O_2 diffusion would limit respiration, according to the method of calculation of Kleiber (1961). Folke & Sestoft (1977) concluded that during the perfusion of rat livers with ouabain there was loss of vascular integrity as shown by increased resistance to perfusion and the appearance of dark areas on the surface of the tissue; this suggests that there was likely impaired delivery of O_2 to the sites of cellular utilization. Since mouse soleus and diaphragm muscles are <0.5 mm thick, our determinations of respiration rates of these whole muscles likely did not involve rate limitation by O_2 diffusion.

Differences in species and tissue sensitivity to ouabain have been established (Schwartz et al. 1969; Allen & Schwartz, 1969). Rat Na^+,K^+-ATPase is particularly insensitive to ouabain largely as a result of instability of the enzyme-ouabain complex which dissociates relatively rapidly. If the mouse enzyme-ouabain complex also dissociates rapidly, the ouabain-sensitive respiration values obtained in this experiment would be minimal indications of active $Na^+ + K^+$ transport in the energy metabolism of mouse soleus and diaphragm muscles.

E. Acknowledgements

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Table I.1 Ouabain-sensitive respiration of whole soleus and diaphragm muscles[1]

Muscle	n	Control	+Ouabain	% Inhibition
Soleus[2]	9	3.7 ± 0.1	2.9 ± 0.1	21.6
Diaphragm[3]	9	4.3 ± 0.1	2.9 ± 0.1	32.6

[1] Values expressed as μl O_2/mg tissue dry wt/h ± SEM.
[2] Average dry wt. 1.4 mg.
[3] Average dry wt. 2.5 mg.

Table I.2 Comparison of ouabain-sensitive respiration of whole and sliced soleus and diaphragm muscles[1]

Treatment	Muscle	n	Control	+Ouabain	% Inhibition
Whole	Soleus[2]	10	3.5 ± 0.1	3.0 ± 0.1	14.3
	Diaphragm[3]	10	4.5 ± 0.1	3.8 ± 0.1	15.6
Sliced	Soleus[2]	10	3.2 ± 0.1	2.5 ± 0.1	21.9
	Diaphragm[3]	10	3.8 ± 0.1	2.8 ± 0.1	26.3

[1] Values expressed as $\mu l\ O_2$/mg tissue dry wt/h ± SEM.
[2] Average dry wt. 1.7 mg.
[3] Average dry wt. 10.2 mg.

II. INHIBITION OF Na^+,K^+-ATPase OF INTACT MOUSE SOLEUS MUSCLE BY Mg^{++}[1]

A. Abstract

The effect of 10 mM Mg^{++} on the inhibition of respiration by ouabain was investigated with intact mouse soleus muscle preparations. Although ouabain caused a 19.7% inhibition of respiration of soleus muscle incubated in 1 mM $MgCl_2$ buffer, the response of respiration to ouabain was abolished upon incubation in buffer containing 10 mM $MgCl_2$. Initial respiration rates were significantly decreased ($p<0.001$) in soleus muscle exposed to 10 mM, as contrasted to 1 mM $MgCl_2$.

B. Introduction

Studies of active Na^+ transport conducted with intact muscle preparations have yielded conflicting results concerning the importance of Na^+,K^+-ATPase (Na^+,K^+-dependent adenosine triphosphatase EC 3.6.1.3.) as a component of cellular energy expenditure. Microcalorimetric studies of the ouabain-sensitive heat production of intact rat (Chinet et al. 1977) and mouse soleus muscles (Biron et al. 1979) have led to the conclusion that active Na^+ transport accounts for no more than 6-8% of resting heat production. However, measurements of the ouabain-sensitive component of

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[1]A slightly modified version of this chapter has been published. Gregg, V. & Milligan, L. P. (1980) Biochem. Biophys. Res. Commun. 95, 608.

O_2 consumption of intact mouse soleus muscle have shown 14-22% of O_2 uptake to be due to the activity of Na^+,K^+-ATPase (Gregg & Milligan, 1980). In view of the key role proposed for Na^+,K^+-ATPase in thyroid thermogenesis (Ismail-Beigi & Edelman, 1970), cold-induced thermogenesis (Guernsey & Stevens, 1977), and as a primary mechanism in the development of obesity (Lin et al. 1978), it is imperative to resolve the current uncertainty concerning the physiological importance of the Na^+,K^+-ATPase in resting energy expenditure.

The microcalorimetric determinations of ouabain-sensitive heat production of mouse soleus muscle were conducted in a buffer containing 10 mM $MgCl_2$ to suppress a secondary rise in heat production which occurred following infusion with ouabain (Chinet et al. 1977; Biron et al. 1979). However, previous investigators have found Na^+, K^+-ATPase activity to be inhibited by a high concentration of Mg^{++} (Bond & Hudgins, 1975; Schwartz et al. 1963). Thus, it has been suggested (Smith & Edelman, 1979) that inhibition by Mg^{++} of the Na^+ pump before challenge with ouabain may have contributed to the lack of inhibition of heat production by ouabain reported by Chinet et al. (1977). The possibility that Mg^{++} will negate inhibition of respiration by ouabain was investigated in this study.

C. Experimental

Adult female mice, in the weight range of 20-25 g, were stunned by a blow to the head and bled from the neck. The intact soleus muscles were removed with care to minimize tissue damage following the procedure of Kohn and Clausen (1971).

Measurement of ouabain-sensitive respiration of intact soleus muscle in control and experimental buffers.

The modified Krebs-Ringer HEPES buffer used as the incubation medium contained (mM): NaCl, 116.8; KCl, 5.9; $NaHCO_3$, 5.0; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $CaCl_2$, 1.0; HEPES (N-(2-hydroxymethylethyl piperazine-N'-2 ethanesulfonic acid), 10.0; $MgCl_2$, 1.0 (control) or 10.0 (experimental); glucose, 5.0; pH 7.3-7.4. Both soleus muscles were utilized from each mouse. Ouabain-sensitive respiration was measured in both the control and experimental buffers for each animal. To achieve thorough oxygenation, muscle preparations were individually equilibrated and incubated in 600 ml beakers containing 25 ml of control or experimental buffer in a shaking water bath at 37°C. Muscle preparations were equilibrated in either control or experimental buffer for 10-20 min and then transferred to the O_2 electrode chamber. O_2 consumption was measured with a YSI O_2 electrode for 10-15 min. The O_2 content of the buffer did not fall below 85% of the initial air-saturated level during the period of measurement. The muscle preparations were then incubated in

control or experimental buffer containing 10^{-3}M ouabain for 45 min and O_2 consumption again measured. Muscle preparations from two mice were incubated in control or experimental buffers for 45 min without ouabain and found to maintain initial respiration rates throughout the incubation period.

Statistical analysis

Respiration rates were compared between treatment groups by the unpaired Student's t-test.

D. Results and Discussion

The initial respiration rate of soleus muscle in the control buffer (4.65 ± 0.39 μl O_2/mg dry wt/h) (Table II.1), was higher than that measured previously for intact mouse soleus muscle (3.7 ± 0.10 μl O_2/mg dry wt/h)(Gregg & Milligan, 1980), in a modified Krebs-Ringer bicarbonate buffer. The ouabain-sensitive component of the respiration of soleus muscle incubated in the control buffer was 19.7%, which is similar to the 14-22% inhibition of respiration reported previously for intact mouse soleus muscle (Gregg & Milligan, 1980). In contrast, using the experimental buffer, addition of ouabain resulted in a slight increase (6%) in the rate of O_2 consumption (Table II.1). This finding is consistent with that obtained by Bond and Hudgins (1975), in which progressive inhibition of red blood cell Na^+,K^+-ATPase was produced with increasing concentrations of Mg^{++} greater

than 3.0 mM in a buffer with a high content of Na^+ and a low content of K^+. Thus, inhibition of respiration by ouabain is abolished in muscle tissue exposed to buffer containing 10 mM $MgCl_2$.

The respiration rate of soleus muscle in the experimental buffer was significantly lower ($p<0.001$) than the initial respiration rate of soleus muscle in the control buffer. The inhibitory effect of Mg^{++} on respiration was significantly greater ($p<0.001$) than the inhibitory effect of ouabain on respiration. It is likely that high Mg^{++} is disruptive to other cell processes involved in energy transformations as well as to Na^+,K^+-ATPase although the mechanism of its inhibitory effect is not known.

We conclude that at least part of the lack of response to ouabain observed in microcalorimetric studies of active Na^+ transport in intact muscle preparations (Chinet et al. 1977; Biron et al. 1979), was due to prior inhibition of the Na^+,K^+-ATPase by the high concentration of Mg^{++} included in the buffer rather than to an unimportant role of active Na^+ transport in the energy expenditure of physiologically intact muscle preparations.

E. Acknowledgements

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Table II.1 Effect of $MgCl_2$ concentration on inhibition of respiration[1] of intact mouse soleus muscle by ouabain.

$MgCl_2$ Concentration	n	Control	+Ouabain	% Change
1.0 mM	12	4.65 ± 0.39	3.75 ± 0.29	-19.7*
10.0 mM	12	3.17 ± 0.21	3.37 ± 0.25	+6.1

[1] Values expressed as μl O_2/mg tissue dry wt/h ± SEM.
* $P<0.001$

III. O_2 CONSUMPTION AND Na^+,K^+-ATPase ACTIVITY IN INTACT SOLEUS MUSCLE FROM COLD EXPOSED MICE

A. Abstract

Rates of O_2 consumption and Na^+,K^+-ATPase activity were measured for intact soleus muscle preparations from warm and cold exposed mice. Cold exposure increased muscle O_2 consumption by 23% ($p<0.001$). The portion (13-14%) of respiration inhibited by 10^{-3}M ouabain did not differ significantly between muscle from warm and cold exposed mice. Increased Na^+,K^+-ATPase activity accounted for 20% of the cold-induced muscle thermogenesis.

B. Introduction

In previous studies of active Na^+-K^+ transport conducted with intact mouse soleus muscle, Na^+,K^+-ATPase (EC 3.6.1.3) activity accounted for 14 to 33% of muscle O_2 consumption (Gregg & Milligan, 1980a,b).

Cold exposure was shown to increase O_2 consumption in sliced preparations of mouse skeletal muscle (Stevens & Kido, 1974) and rat pectoral and diaphragm muscles (Guernsey & Stevens, 1977). Increased energy expenditure at the level of the Na^+,K^+-ATPase accounted for 31-83% of the cold-induced muscle thermogenesis. However, the cell damage resulting from slicing has been shown to cause a decrease in O_2 consumption and tended to cause an increase in the proportion of respiration inhibited by ouabain for soleus

and diaphragm muscles from mice (Gregg & Milligan, 1980a). Therefore, the use of sliced preparations may overestimate the role of the Na^+,K^+-ATPase in cold-induced muscle thermogenesis.

In this experiment, the effect of cold exposure on O_2 consumption and Na^+,K^+-ATPase activity was studied with intact soleus muscle preparations from mice.

C. Experimental

Adult male mice, in the weight range of 20-25g, were housed individually in plastic cages without bedding at either 24°C (warm) or 5°C (cold) for 3 weeks. Standard Purina laboratory chow and water were available ad libitum.

Measurement of O_2 consumption and Na^+,K^+-ATPase activity

Mice were stunned by a blow to the head and bled from the neck. Intact soleus muscles were removed following the procedure of Kohn and Clausen (1971).

The buffer used as the incubation medium contained (mM): NaCl, 116.0; KCl, 5.9; $CaCl_2$, 1.0; NaH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 10.0; HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 20.0 (pH 7.4); glucose, 10.0. Muscles were equilibrated and incubated individually in 600 ml beakers containing 25 ml of buffer in a shaking water bath at 37°C. Muscles were equilibrated in buffer for 5 min and then transferred to the O_2 electrode chamber. O_2 consumption was measured with a YSI

O_2 electrode for 10-15 min. The O_2 content of the buffer did not fall below 85% of the initial air-saturated level during the period of measurement. Muscles were then incubated in buffer containing 10^{-3}M ouabain for 45 min and O_2 consumption was again measured. Percent inhibition of respiration by ouabain was calculated using the ratio of the difference between initial and post-incubation respiration rates to the initial respiration rate.

Statistical analysis

Results are expressed as the mean values and their standard errors. An unpaired Student's t test was used to establish significance of differences between the means for muscle O_2 consumption rate, percent inhibition of respiration by ouabain, and Na^+,K^+-ATPase-dependent and independent respiration.

D. Results and Discussion

Cold-exposure increased ($p<0.001$) muscle O_2 consumption by 23% (Table III.1). This result is consistent with the 9.2-55% increases in O_2 consumption of sliced skeletal muscle preparations from mice and rats when subjected to cold exposure (Stevens & Kido, 1974; Guernsey & Stevens, 1977; Guernsey & Whittow, 1981).

The proportion (13.0-14.1%) of respiration inhibited by ouabain did not differ significantly between treatment groups (Table III.1). This is at the lower end of the

14.3-21.6% inhibition range reported previously for intact mouse soleus muscle (Gregg & Milligan, 1980) and for sliced skeletal muscle preparations from warm and cold exposed mice (Stevens & Kido, 1974).

Muscle from cold exposed mice had a Na^+,K^+-ATPase-dependent respiration value 32% greater ($p<0.001$), and a Na^+,K^+-ATPase-independent value 20% greater ($p<0.001$) than those of warm exposed mice (Table III.1). Increased energy expenditure at the level of the Na^+,K^+-ATPase accounted for 20% of the increased muscle O_2 consumption from cold exposed mice. This value is similar to that reported for sliced mouse skeletal muscle preparations (Stevens & Kido, 1974) but lower than the 54-83% values estimated for the proportion of cold-induced thermogenesis accounted for by Na^+,K^+-ATPase activity for sliced muscle preparations from cold exposed rats (Guernsey & Stevens, 1977; Guernsey & Whittow, 1981). We are not able to conclude that the role of the Na^+,K^+-ATPase in the increased muscle respiration induced by cold exposure differs for mice and rats because the extent to which Na^+-K^+ transport was actually inhibited in the intact soleus muscle preparation under our experimental conditions is not known. The inhibition of respiration by ouabain is a function of both enzyme availability to ouabain and the equilibrium reached between formation and dissociation of the inhibitor-enzyme complex (Tobin & Brody, 1972) as well as the activity of Na^+,K^+-ATPase. Incomplete inhibition of the Na^+,K^+-ATPase

would underestimate the contribution of the Na^+,K^+-ATPase to the increased O_2 consumption observed in the muscle of cold exposed mice. Although our current results indicate that a significant proportion (20%) of the increase in mouse muscle O_2 consumption induced by cold exposure was in support of energy expenditure and heat production at the level of the Na^+,K^+-ATPase, we are not able to support the suggestion of Guernsey & Stevens (1977) that this is the major mechanism of increased thermogenesis.

In conclusion, cold exposure was shown to increase total O_2 consumption of intact soleus muscle preparations from mice. Increased energy expenditure at the level of the Na^+,K^+-ATPase accounted for 20% of the cold-induced increase of muscle O_2 consumption. It is not known how much of the Na^+,K^+-ATPase-independent respiration is due to incomplete inhibition of active Na^+-K^+ transport in the intact mouse soleus muscle preparation or to as yet quantitatively unidentified energy expending metabolic processes.

E. Acknowledgements

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Table III.1 O_2 consumption and Na^+,K^+-ATPase-dependent[1] and independent[2] respiration of intact soleus muscle from warm and cold exposed mice.

Group	Animals	Muscle O_2 consumption (μl O_2/mg/h)	% Inhibition of O_2 consumption by ouabain	Na^+,K^+-ATPase-dependent O_2 consumption (μl O_2/mg/h)[3]	Na^+,K^+-ATPase-independent O_2 consumption (μl O_2/mg/h)[3]
Warm	4	5.20 ± 0.25**a**	13.0 ± 3.8**a**	0.67 ± 0.03**a**	4.52 ± 0.22**a**
Cold	11	6.38 ± 0.39**b**	14.1 ± 2.3**a**	0.90 ± 0.05**b**	5.42 ± 0.33**b**

[1] Na^+,K^+-ATPase-dependent respiration = total O_2 consumption × inhibition by ouabain.
[2] Na^+,K^+-ATPase-independent respiration = total O_2 consumption - Na^+,K^+-ATPase-dependent respiration.
[3] Values expressed as means ± S.E.
a,b Means within a column followed by different letters differ significantly ($p<0.05$).

IV. ROLE OF NA^+,K^+-ATPASE IN MUSCULAR ENERGY EXPENDITURE OF WARM AND COLD EXPOSED SHEEP

A. Abstract

The role of Na^+,K^+-ATPase in the energy expenditure of sheep skeletal muscle and the influence of exposure to cold on this role was studied. An in vitro preparation of muscle was developed which achieved O_2 availability and a functional membrane potential. A 10^{-6} M concentration of ouabain yielded a maximum inhibition of respiration of 38.9 ± 1.8% using muscle preparations from a random group of sheep. Whole body and muscle O_2 consumptions and ouabain-sensitive muscle respiration were measured for warm and cold exposed sheep fed at maintenance or 1150 g alfalfa pellets/d. Cold exposure increased whole body and muscle O_2 consumption. Inhibition of respiration by ouabain was 37.6 ± 1.2% and 41.0 ± 3.6% for warm and cold exposed sheep fed at maintenance, and 28.5 ± 4.0% and 45.0± 4.0% for warm and cold exposed sheep fed 1150 g alfalfa pellets/day. The increase in the ouabain-sensitive component of respiration accounted for 48-79% of the increased O_2 consumption of muscle from cold exposed sheep. It was concluded that the Na^+,K^+-ATPase of sheep muscle is a major means of energy expenditure and has an important role in the increased thermogenesis resulting from cold exposure.

B. Introduction

The activity of Na^+,K^+ATPase (Na^+, K^+-dependent adenosine triphosphatase EC 3.6.1.3) in counteracting the transmembrane movement of Na^+ and K^+ along their concentration gradients has been identified as an important component of cellular energy expenditure, causing 20-45% of the O_2 uptake of resting cells (Whittam, 1961). Activation of Na^+,K^+-ATPase has also been suggested to be an important mechanism for heat production in the cold induced thermogenesis of small mammals (Guernsey & Stevens, 1977). The capacity of the Na^+,K^+-ATPase in basal energy expenditures and heat generation in cold induced thermogenesis was initially studied in sliced tissues from small mammals (see Himms-Hagen, 1976). Concern that damaged cells in the sliced tissue preparations may have yielded physiologically unrealistic impressions of in vivo Na^+,K^+-ATPase activity prompted the use of intact organ preparations including rat liver (Folke & Sestoft, 1977), isolated rat soleus muscle and adipose tissue (Chinet et al., 1977), and mouse soleus and diaphragm muscles (Biron et al., 1979; Gregg and Milligan, 1980). With the intent of achieving further definition of the metabolic components of resting energy expenditure and of energy expenditure in the cold, the objectives of this study were to measure O_2 consumption and Na^+,K^+-ATPase activity in functionally intact sheep muscle preparations and to ascertain the influence of cold exposure of the donor animal, and the

consequent increase in metabolic rate, on these measurements. To achieve these objectives, an in vitro preparation of skeletal muscle was developed in which a functional membrane potential was maintained and there was availability of O_2 to all sites of cellular utilization.

C. Experimental

Muscle preparation

Sheep were anesthetized with 2.0-2.5% halothane gas. A lengthwise incision was made along the neck exposing the sternomandibularis muscle. A longitudinal section of muscle, approximately 5 mm in diameter and 30 mm in length, was tied at each end with 5-0 braided silk suture and bluntly dissected loose in such a way as to prevent leakage from the tied muscle fibers when a cut was made distal to each tie. The muscle section was removed from the animal and placed in ice-cold buffer solution. With the use of a dissecting microscope, the interjacent fascia was removed and the section re-tied into up to six muscle preparations varying in length from 10-25 mm and less than 0.5 mm in thickness, the approximate calculated thickness at which the rate of O_2 diffusion becomes limiting to respiration (Kleiber, 1961).

Electrophysiological studies

The muscle preparations were held on the stage of a fixed stage microscope. Medium was continuously changed by a flow through system supplying Krebs-Ringer bicarbonate

buffer oxygenated with 95%O_2:5%CO_2 at 37°C (Bonkowski & Runion, 1976). The recording microelectrode was mounted on a Prior micromanipulator and cells were penetrated under direct observation. The glass microelectrodes were filled with 3M KCl and connected through a chlorided Ag wire to a WPI M750 electrometer. Electrode resistances were 20-40 MΩ. The D.C. potentials were observed on a Gould Digital Storage Oscilloscope OS4000.

Muscle characterization

Preparations of the sternomandibularis were characterized according to fiber type (Guth and Samaha, 1970). This method identifies muscle fiber types on the basis of qualitative differences in actomyosin ATPase content.

Measurement of sheep muscle respiration and response to ouabain

Muscle preparations were obtained from a random group of eight Suffolk crossbred sheep: four 3-5 year old non-pregnant, non-lactating ewes, two 3-4 year old wethers, and one 2 year old ram. One or two sections were taken per animal per surgery and two to four surgeries were performed per animal. The HEPES buffer used as the incubation medium contained (mM): NaCl, 116.8; KCl, 5.9; $NaHCO_3$, 10.0; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $CaCl_2$, 1.0; HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 20.0

(pH 7.3-7.4); acetate, 5.0; glucose, 10.0. To achieve thorough oxygenation, muscle preparations were equilibrated and later individually incubated in 600 ml beakers containing 25 ml buffer in a shaking water bath at 37°C. Muscle preparations were transferred from the equilibration beaker to the electrode chamber and O_2 consumption measured with a YSI O_2 electrode. Each muscle preparation was incubated for 45 min in one concentration of ouabain within the range of 0 to 10^{-3}M. The rate of O_2 consumption was measured at the end of the incubation period, following which the preparations were dried overnight at 80°C for tissue dry weight determination. Percent inhibition of respiration by ouabain was calculated using the ratio of the difference between initial and that after incubation to the initial respiration rate. Mean percent inhibition of respiration by ouabain was calculated for each concentration of ouabain in which muscle preparations were incubated. A dose response curve was constructed expressing inhibition as a percentage of the maximum inhibition.

Trial 1. Respiration and ouabain-sensitive respiration of muscle preparations from warm and cold exposed sheep fed at maintenance

Eight Suffolk crossbred female sheep, in the weight range of 33-37 kg, were shorn and randomly divided into two groups. Sheep were individually housed in metabolic crates at either 1°C (cold exposed) or 25°C (warm exposed) for 5

weeks prior to muscle sampling. Warm and cold exposed sheep were fed to maintain body weight. Feeding levels were 950 and 1450 g alfalfa pellets/d for sheep exposed to 25°C and 1°C, respectively. Whole animal O_2 consumption was measured using the respiratory gaseous exchange analysis system described by Young et al. (1975). O_2 consumption was measured at 25°C and 1°C for warm and cold exposed sheep, respectively, for a 30 min period 24 h after feeding. Three measurements were made per animal before, during, and after the week of muscle sampling. Respired gases were collected by ventillated hood. Surgery was performed on the cold exposed sheep under warm (25°C) and cold (1°C) conditions. One surgery was performed per animal yielding tissue used to obtain five observations of muscle O_2 consumption and one observation of inhibition of respiration by ouabain at each concentration of inhibitor. Values for percent inhibition of muscle respiration by ouabain were determined as described. Muscle preparations were exposed to ouabain in the range of 0 to 10^{-5}M since ouabain concentrations of 10^{-6}M or greater were shown to yield maximum inhibition of respiration for excised sheep muscle (Fig. 1). Dose response curves were constructed for each animal.

Trial 2. Respiration and ouabain-sensitive respiration of muscle preparations from warm and cold exposed sheep fed at the same level of feed intake

The same experimental outline was followed as in Trial 1 with the same experimental animals except that all sheep were given 1150g/day of the pelleted alfalfa diet and all surgeries were performed under warm (25°C) conditions.

Na^+,K^+-ATPase Assay

The Na^+,K^+-ATPase activity of muscle homogenates was measured for sheep of trials 1 and 2 according to a modification of the method of Lo et al.(1976). Samples of the sternomandibularis muscle, approximately 30 mg in weight, were taken from animals during surgery and immediately frozen in liquid N_2. Frozen muscle samples were homogenized in 10 volumes of the homogenizing medium with a Tekmar homogenizer. Crude homogenate (0.1 ml) was added to 0.9 ml of the reaction mixture in the presence and absence of 0.02 M KCl. After incubation, the inorganic phosphate (Pi) content of the reaction mixture was determined by the method of Fiske & Subbarow (1925). Na^+,K^+-ATPase activity was measured as the difference between Pi generated in the presence and absence of K^+. The protein concentration of the muscles was determined by the method of Lowry et al.(1951).

Statistical analysis

Values for Ki, the concentration of ouabain giving one-half maximal inhibition of Na^+,K^+-ATPase-dependent respiration, were calculated from a least-squares regression line fitted through the linear portion of the individual dose response curves for the ouabain effect on muscle respiration. Values for Ki and means and their standard errors for total, ouabain-sensitive and ouabain-insensitive respiration, and Na^+,K^+-ATPase activities were evaluated for statistical differences by the unpaired Student's t-test (Steel & Torrie, 1960).

D. Results

Halothane gas was used as the surgical anaesthetic since it has been shown in isolated nerve-muscle preparations that membrane potentials regain pre-exposure status shortly after removal of the volatile gas (MacGregor, 1978). Membrane potentials, measured during development of the intact muscle technique, were -50 mV, or more negative. The sternomandibularis of sheep is a predominantly red type muscle and contained 66% red and 33% white fibers; intermediate type fibers were not present.

Response of sheep muscle respiration to ouabain

During the course of a day's measurements, some of the muscle preparations were held in the equilibration beaker for up to 3 h before measurement of initial rate of O_2

consumption. No consistent decrease in the rate of O_2 consumption occurred during this period. The mean initial respiration rate of the sheep muscle preparations was 2.65 ± 0.11 μl O_2/mg dry wt/h (n=34). The dose response curve constructed for ouabain inhibition of respiration of sheep muscle is shown in Fig. IV.1. The rate of O_2 consumption of control muscle preparations incubated in buffer without ouabain for 45 min did not change ($P>0.05$) from the initial rates of respiration. The lowest concentration of ouabain resulting in maximum inhibition of respiration was 10^{-6}M. Values for inhibition of respiration at greater concentrations of ouabain were not different ($P>0.05$) from those obtained with 10^{-6}M ouabain. Inhibition by 10^{-6} M ouabain was 38.9 ± 1.8% of the total respiration of the muscle. The calculated Ki value was 0.25 μM.

Trial 1. Respiration and ouabain-sensitive respiration of muscle preparations from warm and cold exposed sheep fed at maintenance

The body weights of both the warm and cold exposed sheep were maintained throughout the experimental period. Whole animal O_2 consumption rates were 8.9 ± 1.0 and 12.6 ± 1.0 l O_2/h, for warm and cold exposed sheep, respectively (Table IV.1). The increased metabolic rate of the cold exposed sheep was reflected in the initial respiration rates of their muscle preparations; the respiration rates were 1.82 ± 0.11 and 2.69 ± 0.16 μl O_2 /mg dry wt/h for the

preparations from warm and cold exposed sheep, respectively. Ouabain-sensitive respiration was 37.6 ± 1.2% and 41.0 ± 3.6% of total respiration in the muscles of warm and cold exposed sheep, respectively. Both the ouabain-sensitive and ouabain-insensitive components of respiration of muscle preparations were significantly increased ($P<.001$) by cold exposure of sheep fed at maintenance; there was an increase of 69% in the ouabain-sensitive component, while the ouabain-insensitive component increased 39% (Fig. IV.2). The increase in ouabain-sensitive respiration accounted for 48% of the cold induced increase in muscle respiration. The dose response curves for the warm and cold exposed groups are shown in Fig. IV.3. Ki values were calculated for each animal; the mean Ki did not differ significantly ($p>0.05$) between treatment groups. The overall mean Ki for the ouabain inhibition of muscle respiration for preparations from the warm and cold exposed sheep was 0.19 ± 0.06 μM.

Trial 2. Respiration and ouabain-sensitive respiration of muscle preparations from warm and cold exposed sheep fed at the same level of intake

The warm exposed sheep gained an average of 37 g/d and the cold exposed sheep lost an average of 33 g/d during the experimental period while receiving 1150g alfalfa pellets per day. Whole animal respiration rates were 11.1 ± 1.0 and 13.8 ± 1.0 l O_2/h, for warm and cold exposed sheep, respectively (Table IV.2). Surgeries were performed under

warm (25°C) conditions since in two instances of doing surgery in the cold in Trial 1, difficulties of surgery were increased and no effect of surgery room temperature on initial and ouabain-sensitive respiration was observed. Initial respiration rates were 2.09 ± 0.17 and 3.09 ± 0.19 μl O_2/mg dry wt/h for the muscle preparations from the warm and cold exposed sheep, respectively. Ouabain-sensitive respiration was 28.5 ± 4.0% of total respiration in the warm exposed sheep and 45.0 ± 4.0% in the cold exposed sheep. The ouabain-sensitive component of respiration of muscle preparations was increased 132% in cold exposed sheep, accounting for 79% of the total increase in muscle respiration due to cold exposure (Fig. IV.2). The ouabain-insensitive component of respiration did not differ ($P>0.05$) between warm and cold exposed sheep fed at the same level of intake. The mean dose response curves for the warm and cold exposed groups are shown in Fig IV.4. Ki values did not differ ($P>0.05$) between treatment groups; the overall mean Ki value of warm and cold exposed sheep fed at the same level of intake was 0.20 ± 0.03 μM.

Na^+,K^+-ATPase assay

In animals fed at maintenance and at the same level of intake, the mean Na^+,K^+-ATPase activity of muscle from warm exposed sheep (1.31 ± 0.26 μmole Pi/mg protein/h) did not differ ($P>0.05$) from that of muscle from cold exposed sheep (1.48 ± 0.26 μmole Pi/mg protein/h).

E. Discussion

The difficulty of obtaining tissue slice preparations of muscles from large animals which would not contain a large proportion of damaged cells prompted the development of a preparation that more closely resembled the in vivo muscle state, for example, by maintaining a functional membrane potential. The small size of this muscle preparation allowed repeated sampling of each animal and avoided problems associated with ouabain perfusion of whole organ preparations, such as loss of vascular integrity and oxygenation (Folke & Sestoft, 1977).

Ouabain, the specific inhibitor of Na^+,K^+-ATPase, inhibited 40% of the respiration of excised sheep muscle, supporting the role of Na^+,K^+-ATPase as a major component of cellular energy expenditure. This measurement is in agreement with values reported for the ouabain-sensitive respiration of rat diaphragm (Asano et al. 1976; Ismail-Beigi & Edelman, 1970) and mouse soleus and diaphragm muscles (Gregg & Milligan, 1980). The sigmoidal dose response curves obtained for ouabain inhibition of the Na^+,K^+-ATPase-dependent respiration of sheep muscle (Figs. IV.3, IV.4), are similar to the pattern reported for purified Na^+,K^+-ATPase preparations from other mammals (Charnock & Simonson, 1977, 1978).The Ki value of 0.25 μM calculated for ouabain inhibition of respiration of muscle from a random group of sheep is within the range of 0.11-2.50 μM reported for purified Na^+,K^+-ATPase

preparations for several mammalian tissues (Charnock & Simonson, 1977, 1978; Tobin & Brody, 1972).

Cold induced thermogenesis, as studied in small mammals, is thought to be a consequence of increased heat production by brown adipose tissue (Horwitz, 1979). The increased thermogenesis induced by cold exposure appears to be mediated by a complex interaction involving neural and hormonal factors and includes increased thyroid activity. (Horwitz, 1979). In larger animals containing little, if any, brown adipose tissue, muscle becomes an important site of heat production when an increased thermal demand is imposed (Jansky, 1973). Indeed, we found that the rate of O_2 uptake of muscle preparations from cold exposed sheep was greater than uptake by preparations from warm exposed sheep. It is then of importance to ascertain the mechanisms within the tissue by which the increased metabolic rate is achieved. The results obtained using functionally intact preparations indicate that 48-79% of the increased O_2 consumption observed in muscle preparations from cold exposed sheep is attributable to increased Na^+, K^+-ATPase activity. In principle, this is in agreement with the evidence derived with damaged muscle preparations that a major portion of the increased O_2 consumption of muscle tissue from cold exposed animals results from increased energy expenditure by means of Na^+,K^+-ATPase activity (Guernsey & Stevens, 1977; Stevens & Kido, 1974).

The mechanism of enhancement of Na^+,K^+-ATPase activity of muscle from cold exposed animals is not clear at this time. Stimulation of the activity of pre-existing enzyme or hormonal induction of increased enzyme synthesis or of the synthesis of a more active enzyme are possible ways of achieving the observed increase of the Na^+,K^+-ATPase activity of muscle from cold exposed animals. There is evidence of increased thyroid activity in sheep subjected to cold stress (Westra & Christopherson, 1976) and ^{3}H-ouabain binding studies have indicated increased amounts of Na^+,K^+-ATPase in the muscles of rats upon treatment with thyroid hormone (Lin & Akera, 1978), thus one might expect increased amounts of Na^+,K^+-ATPase in the skeletal muscle of cold exposed sheep. However, overall Na^+,K^+-ATPase activities, measured in this study as an index of the maximum capacity of the enzyme system, did not differ significantly ($p>0.05$) between warm and cold exposed sheep. The present study does not provide conclusive evidence for resolution of the possibilities of increased activity of existent enzyme versus an increased amount of enzyme as the cause of the increase in Na^+,K^+-ATPase-dependent respiration in the muscle of cold exposed sheep.

The kinetic constant Ki, a measure of enzyme sensitivity to inhibitor, may be a useful tool in the assessment of changes in enzyme characteristics occuring with different physiological stresses. The lack of difference between calculated Ki values from warm and cold

exposed sheep fed at maintenance or at the same level of intake may indicate a lack of change in the nature of the enzyme in the cold exposed animals.

In designing a study of effects of cold stress on animals, an investigator is faced with the dilemma of providing additional dietary energy intake to the cold exposed animals to offset the imposed thermal demand, or of simply providing the same amount of feed to the two experimental groups. The former choice results in warm and cold exposed groups at differing levels of intake, as in Trial 1, while the latter choice results in warm and cold exposed groups that differ quantitatively in the energy they can expend on metabolic processes other than those expressly for heat production, as in Trial 2, in which the cold exposed group lost weight. Thus, to arrive at a reliable conclusion, it is of importance to have examined effects under both of the foregoing experimental circumstances. Considerable confidence arises upon having obtained similar findings regarding Na^+,K^+-ATPase activity under both experimental circumstances. It is concluded that expenditure of energy by Na^+,K^+-ATPase-catalyzed transport of Na^+ and K^+ across the plasma membrane, has a highly significant involvement in the basal energy expenditure of sheep muscle and that a major part of the increased respiration, and presumably heat generation, of muscle from cold exposed sheep was due to increased Na^+,K^+-ATPase activity.

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Table IV.1 Whole animal and muscle respiration from sheep fed at maintenance[1].

Treatment	Whole animal O_2 consumption (l O_2/h)	Muscle O_2 consumption (μl O_2/mg/h)	% Inhibition by ouabain of muscle O_2 consumption[2]
Warm	8.9 ± 1.0	1.82 ± 0.11	37.6 ± 1.2
Cold	12.6 ± 1.0*	2.69 ± 0.16**	41.0 ± 3.6

[1] Maintenance feed intakes were 950 and 1450 g alfalfa pellets/d for warm and cold adapted sheep, respectively.
[2] ouabain=10^{-6}M.
* $P<0.05$
** $P<0.001$

Table IV.2 Whole animal and muscle respiration from sheep fed at the same level of intake[1].

Treatment	Whole animal O_2 consumption (l O_2/h)	Muscle O_2 consumption (μl O_2/mg/h)	% Inhibition by ouabain of muscle O_2 consumption[2]
Warm	11.1 ± 1.0	2.09 ± 0.17	28.5 ± 4.0
Cold	13.8 ± 1.0	3.09 ± 0.19**	45.0 ± 4.0**

[1] 1150 g alfalfa pellets/d
[2] ouabain=10^{-6}M.
** P<0.001

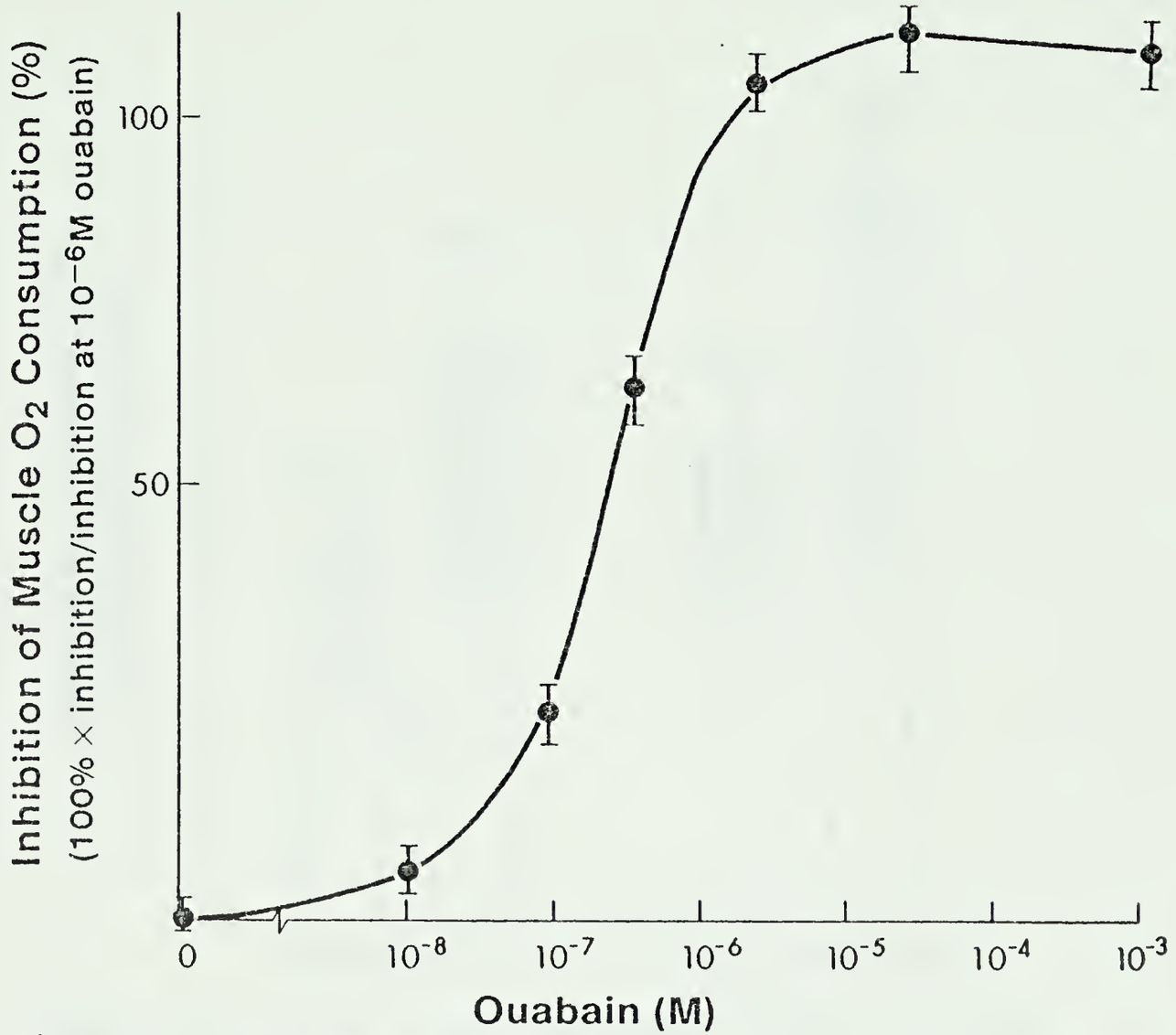

Figure IV.1. Relative inhibition of sheep muscle respiration by ouabain expressed as a percentage of inhibition at 10^{-6}M ouabain (maximum inhibition). Values are means ± S.E.

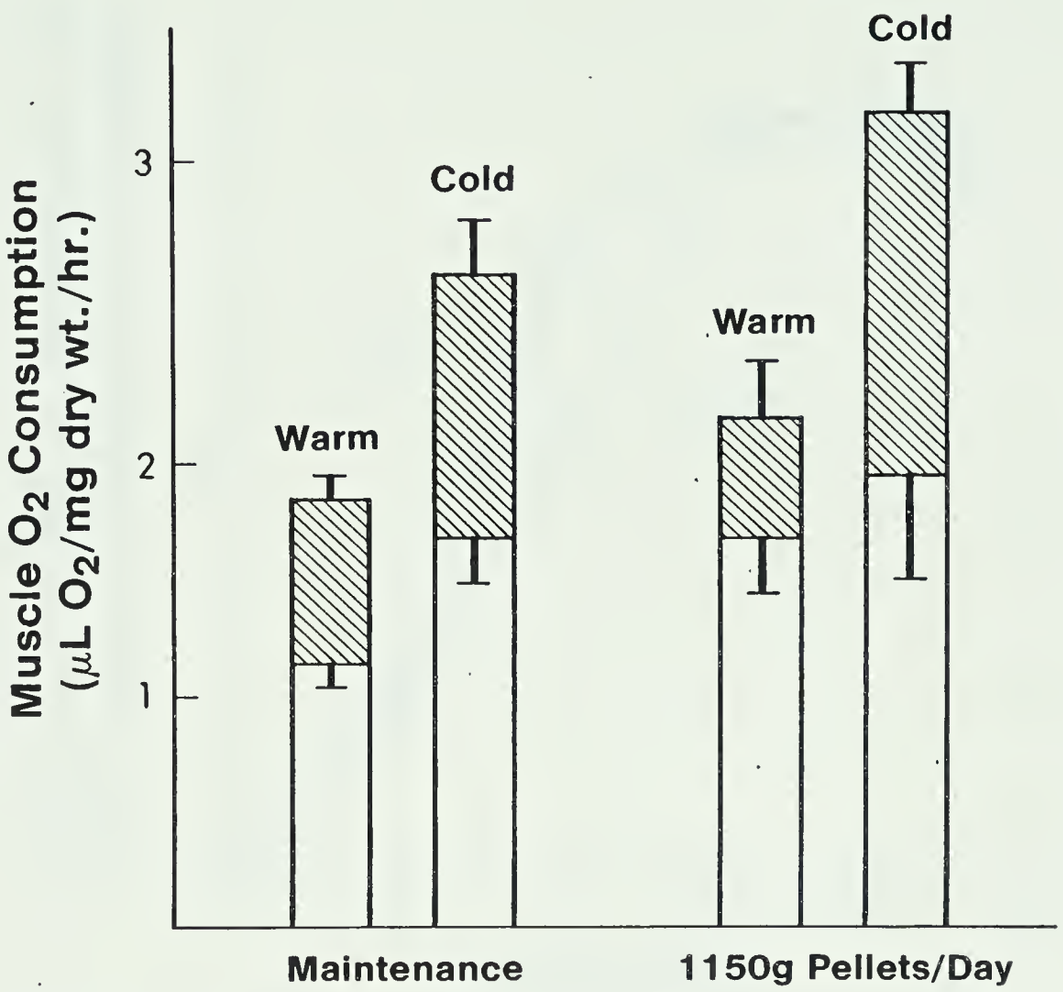

Figure IV.2. Total respiration and ouabain-sensitive (hatched) and insensitive (clear) respiration of muscle from warm and cold exposed sheep fed at maintenance or 1150 g alfalfa pellets/d. Values are means ± S.E.

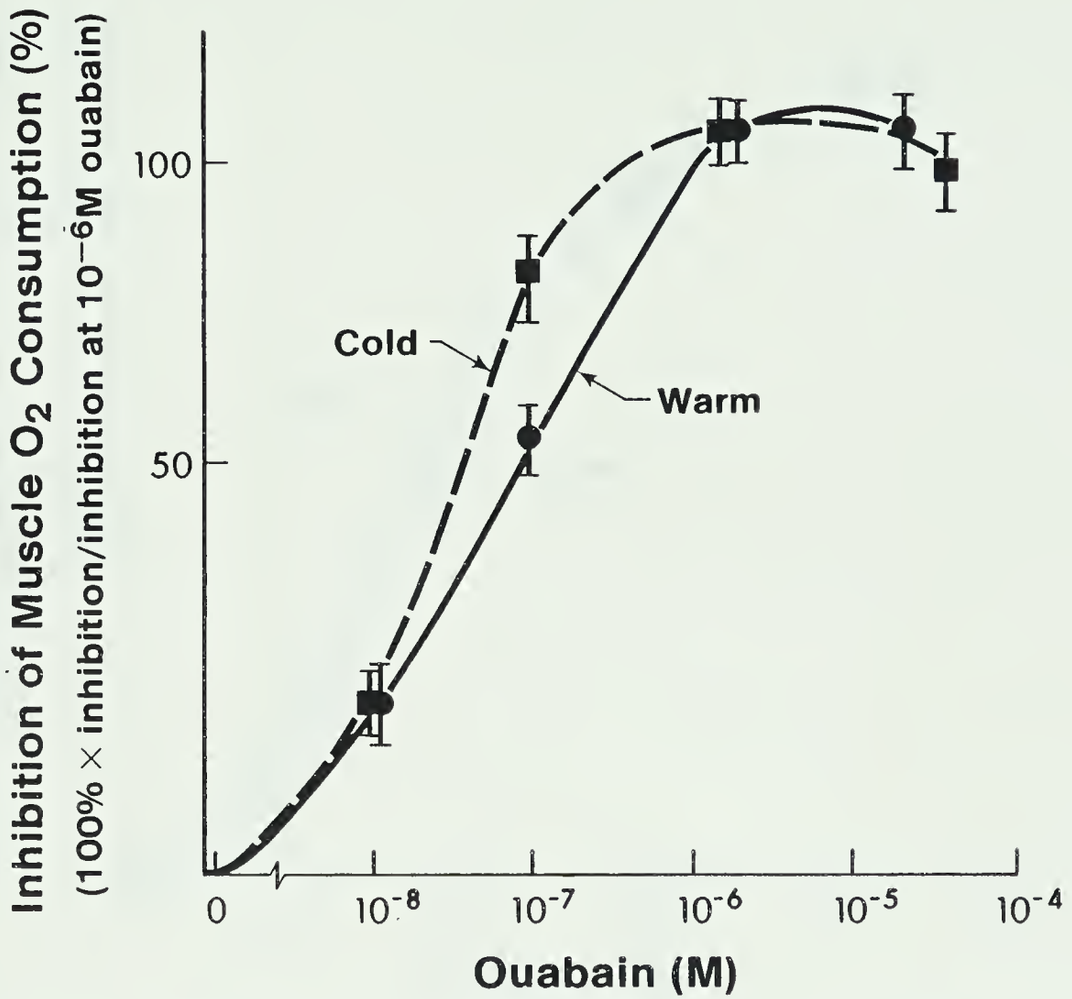

Figure IV.3. Relative inhibition by ouabain of respiration of muscle from warm and cold exposed sheep fed at maintenance. Inhibitions expressed as a percentage of inhibition at 10^{-6}M ouabain (maximum inhibition). Values are means ± S.E.

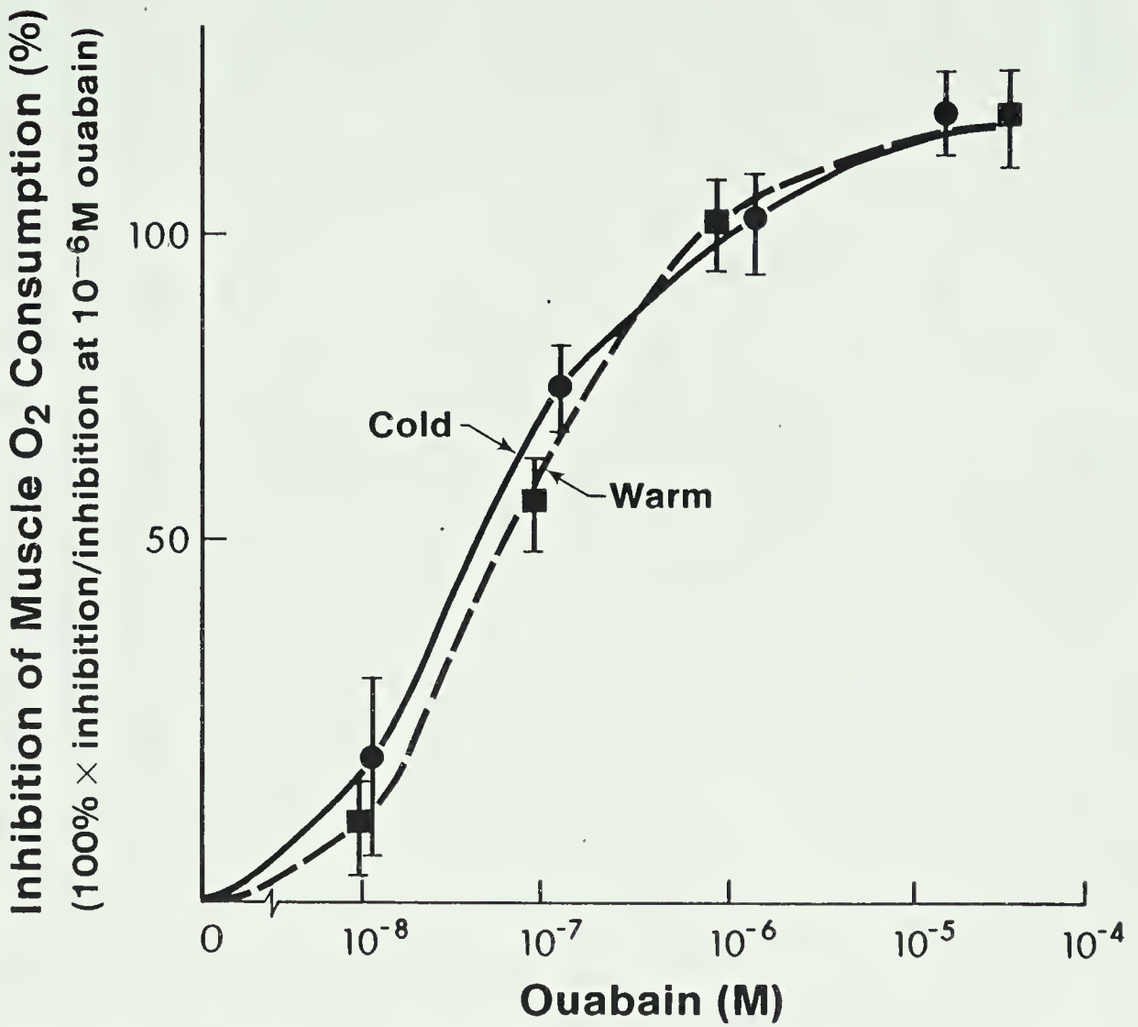

Figure IV.4. Relative inhibition by ouabain of respiration of muscle from warm and cold exposed sheep fed 1150 g alfalfa pellets/d. Inhibitions expressed as a percentage of inhibition at 10^{-6}M ouabain (maximum inhibition). Values are means ± S.E.

V. ENERGY COSTS OF NA^+,K^+ATPase ACTIVITY AND PROTEIN SYNTHESIS IN MUSCLE FROM CALVES DIFFERING IN AGE AND BREED

A. Abstract

An in vitro preparation was used to measure rates of O_2 consumption, Na^+,K^+-ATPase-dependent respiration, ^{14}C-phenylalanine incorporation and tyrosine release of skeletal (sternomandibularis) muscle from 10-21d and 7 month dairy calves, and control and extreme double-muscled (DM) calves. Rate of O_2 consumption was greatest ($p<0.001$) for muscle from 10-21d dairy calves and lowest ($p<0.05$) for control DM calves. Ouabain (10^{-5}M) caused a 40% inhibition of muscle respiration. Na^+,K^+-ATPase-dependent respiration was similar for muscle from all calf groups except 10-21d dairy calves which had a value 26% greater ($p<0.001$) than that of older dairy calves. Na^+,K^+-ATPase-independent respiration was 16% greater ($p<0.001$) for muscle from 10-21d than that of older dairy calves while muscle from extreme DM calves had a value 11% greater than that of control DM calves. The rate of ^{14}C-phenylalanine incorporation was greater ($p<0.05$) for muscle from 10-21d dairy than from older dairy calves, similar between older dairy and control DM calves, and decreased ($p<0.05$) for extreme DM calves. Rate of tyrosine release was greatest ($p<0.05$) for muscle from control and extreme DM calves; both dairy groups had similarly low rates of muscle tyrosine release. The energy estimated to be required for peptide bond synthesis

accounted for 2.0 to 3.3% of the O_2 consumption of the muscle preparations.

B. Introduction

In order to more fully understand whole animal energy expenditure it is necessary first to identify the causes of metabolic energy expenditure, and then to determine their quantitative importance under a variety of physiological conditions.

Active Na^+-K^+ transport, that is, the activity of the plasma membrane Na^+,K^+-ATPase (EC 3.6.1.3) in counteracting transmembrane movement of Na^+ and K^+ along their concentration gradients, has been suggested to be a major component of the energy expenditure of animals and has been estimated to account for 20-45% of the O_2 uptake of resting cells (Whittam, 1961). Protein synthesis has also been suggested to be a major energy cost of animals, accounting for up to 30% of the heat production of cattle (Lobley et al. 1980). The extent to which energy expended by processes such as active Na^+-K^+ transport and protein synthesis can vary between animals and is influenced by genetic and environmental factors is not clear. Evidence that exposure of animals to a cold environment selectively increased energy expenditure at the level of the Na^+,K^+-ATPase has been presented for muscle preparations from sheep (Chapter IV).

The objectives of this experiment were to obtain physiologically realistic estimates of the magnitudes of the energy costs of active Na^+-K^+ transport and protein synthesis in skeletal muscle from calves and to examine the effects of breed and age upon the relative costs of these two processes as components of background or maintenance energy expenditure of the tissue.

C. Experimental

Animals

Muscle samples were obtained from six 7 month old male calves from a beef crossbred population selected for a high incidence of double-muscling (DM), two calves exhibited normal muscling (control DM) and four calves exhibited overt muscular hyperplasia (extreme DM); three 10-21d old male dairy (Holstein) calves; and three 7 month old male dairy calves. All animals were housed indoors in heated barns (approximately 20°C) for at least 4 weeks prior to surgery. The 10-21d dairy calves were fed a milk replacer diet; 7 month calves were fed good quality grass hay free choice and a ration of barley-oat concentrate mix containing minerals. Water was available ad libitum. Animals were fasted overnight prior to surgery.

Respiration and Na^+,K^+-ATPase-dependent respiration

A section of the sternomandibularis muscle was taken from each animal and preparations made following the method

of Gregg & Milligan (Chapter IV). One surgery was performed per animal yielding tissue used to obtain four observations of muscle O_2 consumption and one observation of inhibition by ouabain at each concentration of inhibitor. Immediately upon removal from the animal, muscle sections were placed in cooled (15°C) HEPES buffer containing 10 mM glucose and 5 mM acetate as substrates, and the small tied fibre bundles (approximately 20.0 x 0.5 mm) were prepared at room temperature with the aid of a dissecting microscope. Following measurement of initial respiration rates (Chapter IV) in an O_2 electrode system, the muscle preparations were incubated in buffer containing 0 to 10^{-4}M ouabain. Respiration rates were then measured again. Percent inhibition of respiration by ouabain was calculated using the ratio of the difference between initial and post-incubation respiration rates to the initial respiration rate.

Measurement of ^{14}C-phenylalanine incorporation

Muscle sections were placed in HEPES buffer to which the following additions had been made: essential and non-essential amino acids, except phenylalanine, at the concentrations reported for sheep plasma (Bergman et al. 1974); phenylalanine, 500 μM; insulin, 0.1 unit per ml; chloramphenicol, 0.3 mg per l. Four muscle preparations from each animal were incubated in 3 ml of the complete HEPES buffer containing approximately 0.28 μCi of L-(U-^{14}C)-

phenylalanine (Amersham Corp., Ontario) per ml, two preparations were incubated for 1.0 h and two for 2.5 h in a shaking water bath at 37°C. Preliminary studies established the rate of ^{14}C-phenylalanine incorporation to be linear for incubations periods of up to 3.0 h. At the end of the incubation period the muscle preparations were rinsed, blotted and weighed. They were then homogenized in 1 ml of cold 50% (w/v) trichloroacetic acid, centrifuged, and the precipitate washed according to the method of Fulks et al. (1975). Acid-precipitated pellets were combusted in a Beckman biological material oxidizer and $^{14}CO_2$ collected in 10 mls of CO_2-trapping cocktail (50%, v/v, toluene, 30% methyl cellosolve, 20% monoethanolamine, 5.0 g PPO, 0.2 g POPOP). Radioactivity was measured with a Searle Mark III liquid scintillation counter and counting efficiency determined with the channels-ratio method.

In a separate experiment, acid-precipitated material from muscle preparations incubated 2.5 h was dried, hydrolyzed and chromatographed according to the method of McBride et al. (1979). Sections of the thin layer chromatography plates were scraped into separate counting vials and counted as described above. Radioactivity was found to be present only at the position corresponding to the phenylalanine standard.

Measurement of tyrosine release

The rate of tyrosine release from muscle was measured in the HEPES buffer used for the study of ^{14}C-phenylalanine incorporation, in which was included 0.5 mM cycloheximide and from which tyrosine was ommitted, according to the method of Fulks et al. (1975). Two muscle preparations from each animal were pre-incubated in 2 ml of buffer in a shaking water bath at 37°C for 0.5 h then transferred to similar flasks; one preparation was incubated for 0.5 h and the other for 1.5 h. At the end of the incubation period, muscle preparations were blotted and weighed. The amount of tyrosine in the buffer was measured fluorometrically by the method of Waalkes & Udenfriend (1957). Preliminary studies established the rate of tyrosine release from muscle preparations into buffer to be linear for incubation periods of up to 2.5 h after a 0.5 h pre-incubation period.

Statistical analysis

Results are expressed as the mean values and their standard errors. An unpaired Student's t test was used to establish the significance of differences between the means for muscle O_2 consumption, percent inhibition of respiration by 10^{-5}M ouabain, and Na^+,K^+-ATPase-dependent and independent respiration. The rates of ^{14}C-phenylalanine incorporation and tyrosine release were determined by least squares analysis. Differences between groups were located by the Student-Newman-Kuel range test (Steel & Torrie, 1960).

D. Results

O_2 consumption and Na^+,K^+-ATPase activity

Total muscle O_2 consumption and the proportion of respiration inhibited by 10^{-5}M ouabain are shown in Table V.1.

The rate of muscle O_2 consumption was greatest ($p<0.05$) for 10-21d dairy calves and was reduced by 16% in the muscle from the older dairy calves. Among calves of similar age, dairy calves had a muscle O_2 consumption rate greater ($p<0.05$) than control DM calves.

Dose-response curves constructed for each calf group differed in absolute values for percent inhibition of respiration observed at each concentration of inhibitor but had a similar sigmoidal shape and a similar value for the lowest concentration of inhibitor yielding maximum inhibition (10^{-5}M ouabain).

The proportion of respiration inhibited by ouabain ranged from 39.4 ± 2.9% for older dairy to 42.7 ± 1.4% for control DM calves. Na^+,K^+-ATPase-dependent respiration, the amount of O_2 calculated to have been consumed to support the ouabain-inhibitable portion of Na^+,K^+-ATPase activity, was statistically similar for muscle from all calf groups except 10-21d dairy calves which had a value 26% greater ($p<0.001$) than that of older dairy calves.

Na^+,K^+-ATPase-independent respiration, the residual portion of muscle O_2 consumption not accounted for by activity of the Na^+,K^+-ATPase, was greatest for muscle from

10-21d dairy calves. The value for muscle from 10-21d dairy calves was 16% greater ($p<0.001$) than that of older dairy calves. For calves of similar age, Na^+,K^+-ATPase-independent respiration was measured to be 11% greater ($p<0.05$) for muscle from extreme than that of control DM calves and 19% greater ($p<0.005$) for muscle from older dairy than that of control DM calves.

Rates of ^{14}C-phenylalanine incorporation and tyrosine release

Rates for ^{14}C-phenylalanine incorporation into muscle protein are shown in Table V.2. The rate of ^{14}C-phenylalanine incorporation into muscle differed ($p<0.05$) between all groups except between older dairy and control DM calves. Ks values ranged from 0.7 to 1.5%/d (Table V.2); muscle preparations from 10-21d dairy calves had a Ks value 50% greater than those from older dairy calves, and muscle preparations from control DM calves had a Ks value 54% higher than those from extreme DM calves. The Ks values for muscle preparations from older dairy and control DM calves were similar. The estimated cost of incorporation of amino acids into growing peptide chains ranged from 2.0 to 3.3% of the total in vitro O_2 consumption of the muscle preparations (Table V.2).

Rates of release of tyrosine from muscle preparations are shown in Table V.3. The rate of tyrosine release from muscle differed ($p<0.05$) between all calf groups except

between 10-21d and older dairy calves. Both dairy groups had tyrosine release rates which were too low to allow accurate measurement. Muscle preparations from extreme DM calves had a greater ($p<0.05$) rate of tyrosine release (Table V.3) than those from control DM calves.

The fraction of muscle protein degraded per day (Kd) was calculated assuming a muscle tyrosine content of 2.8 mole % (Chang & Goldberg, 1978). Kd values thus calculated were 0.1%/d for 10-21d and older dairy calves, 0.8%/d for control DM calves, and 5.4%/d for extreme DM calves. It was assumed that tyrosine released from muscle originated from muscle protein since Fulks et al. (1975) have shown that under similar experimental conditions muscle free tyrosine content did not change during 3 h of incubation.

E. Discussion

Exposure of muscle to 10^{-5}M ouabain resulted in an average of 40% for inhibition of respiration. This value is in agreement with values for inhibition of respiration by ouabain reported for skeletal muscle from sheep (Chapter IV), mice (Gregg & Milligan, 1980a), and rats (Asano et al., 1976; Ismail-Beigi & Edelman, 1970).

The rates of O_2 consumption measured for calf muscle preparations were similar to those previously measured with like preparations of the same muscle for adult sheep (Chapter IV). A decrease of metabolic rate with age has been previously observed for cattle (Webster et al. 1974). The

greater rate of muscle O_2 consumption measured for 10-21d than for older dairy calves resulted from increases in both the Na^+,K^+-ATPase-dependent and independent components of respiration. For calves of similar age, the differences measured for total O_2 consumption between extreme and control DM calves and between dairy and control DM calves were due to an increased amount of O_2 consumed in the Na^+,K^+-ATPase-independent component of respiration.

The use of ouabain to determine Na^+,K^+-ATPase-dependent respiration was criticized (Himms-Hagen, 1976) primarily on the basis that the decreased O_2 consumption observed in the presence of ouabain may be due to altered intracelluar Na^+ and K^+ concentrations and may, therefore, be secondary to the inhibition of the Na^+,K^+-ATPase. However, Asano et al. (1976) found measurements of the ouabain-inhibitable O_2 consumption of rat skeletal muscle to be independent of a wide range of experimentally induced changes in intracellular Na^+ and K^+ concentrations. Also, inhibition by ouabain of the rate of glycolytic substrate-level phosphorylation in ascites tumor cells was shown to have resulted from direct inhibition of Na^+,K^+-ATPase rather than from a disturbance of glycolytic enzyme function through alterations of intracellular K^+ concentrations (Scholnick et al. 1973). A second objection (Chinet et al. 1977) concerned the use of sliced tissue preparations for measurement of Na^+,K^+-ATPase-dependent respiration: rapid leakage of Na^+ into the cell at sites of membrane damage may have occurred

and caused a non-physiological stimulation of the Na^+,K^+-ATPase leading to the large values measured for Na^+,K^+-ATPase-dependent respiration. Investigations conducted with intact organ perfusions have measured Na^+, K^+-ATPase-dependent respiration or heat production to account for less than 6% of total tissue O_2 consumption or heat production (Chinet et al., 1977; Folke & Sestoft, 1977). In contradiction to this criticism, Gregg & Milligan (1980b) have presented evidence that at least part of the low response to ouabain obtained in the microcalorimetric studies of perfused organs was due to prior inhibition of the enzyme by the experimental conditions.

The importance of tissue preparation and condition in achieving measurements indicative of physiological O_2 consumption and Na^+,K^+-ATPase activity cannot be ignored. The muscle preparation used in this experiment was developed specifically to study the metabolism of muscle from large mammals under conditions which minimize cellular damage and maintain physiological characteristics such as membrane potential and oxygenation (Chapter IV). The inhibition of respiration induced by ouabain is a function of both enzyme availability to ouabain and the equilibrium reached between formation and dissociation of the inhibitor-enzyme complex (Tobin & Brody, 1972). The proportion of Na^+,K^+-ATPase activity actually inhibited in the muscle preparation under our experimental conditions is not known. Therefore, our measurements of the proportion of muscle O_2 consumption

required for support of Na^+,K^+-ATPase activity must be considered as minimum estimates.

Accurate calculation of rates of protein synthesis from incorporation of ^{14}C-phenylalanine into muscle protein requires that the specific activity of phenylalanine-tRNA be known. McKee et al. (1978) have shown for perfused rat heart that the specific activities of extracellular, intracellular, and tRNA-bound phenylalanine are the same when the perfusate phenylalanine concentration was 0.4 mM or greater. It was assumed that protein degradation that may have occurred during the period of incubation did not significantly influence the estimate of synthesis by causing cleavage of newly added phenylalanine from peptide chains.

The calculated values for the fraction of muscle protein synthesized per day (Ks)(Table V.2) are in good agreement with Ks values reported for heifer and cow muscle (0.8-2.0%/d) by Lobley et al. (1980, 1978) as determined by in vivo constant infusion of ^3H-tyrosine. The greater rate of muscle protein synthesis measured for 10-21d dairy than from older dairy calves is consistent with the higher Ks values for protein synthesis measured for immature in contrast to adult animals (see Garlick, 1980).

Although the rate of protein synthesis was 50% greater in muscle of 10-21d dairy calves than that of older dairy calves, the increased rate of peptide bond synthesis would account for only 9% of the difference in total muscle O_2 consumption measured between the two dairy groups. It is not

known how much of the greater Na^+,K^+-ATPase-dependent respiration might have been in support of, or association with, the increased rate of protein synthesis as suggested by Reeds et al. (1980). Older dairy and control DM calf muscle did yield similar values for both the amount of O_2 consumed in direct support of protein synthesis and Na^+,K^+-ATPase-dependent respiration. However, among 7 month old calves, the rate of protein synthesis in the muscle of control DM calves was 55% greater than in muscle of extreme DM calves while no difference was measured for Na^+,K^+-ATPase-dependent respiration between the two calf groups. These results do not appear to support a close association of increased rate of protein synthesis and of Na^+,K^+-ATPase activity. However, the extent to which Na^+,K^+-ATPase-dependent respiration is underestimated and the extent to which differences in protein degradation may have affected muscle O_2 consumption are not known. Therefore, it is not possible to reach a conclusion regarding a relationship between rate of protein synthesis and Na^+,K^+-ATPase activity.

Rates of protein turnover should not be calculated from our results since the rates of ^{14}C-phenylalanine incorporation and of tyrosine release were measured under different experimental conditions, the latter in a medium with tyrosine omitted and cycloheximide added. Nonetheless, estimated rates of protein degradation (Table V.3) were less than, or similar to, rates of protein synthesis (Table V.2)

for muscle preparations from all calf groups except extreme DM calves. The DM muscle preparations exhibited an enhanced rate of tyrosine release which is certainly suggestive of an increased capacity for protein degradation.

DM cattle appear to partition energy in a way that is different from non-double muscled cattle including altered potentials for protein and fat deposition (Holmes & Ashmore, 1972). The metabolic cause of these changes is not known. One might speculate, on the basis of the possibility of increased protein degradation, that muscles of extreme DM calves may expend more energy for protein turnover than those of control DM calves and this might account for the tendency for increased total muscle O_2 consumption. In the animal, increased energy expenditure for such a maintenance function could result in a reduction of energy available for fat deposition.

The available evidence supports the importance of activity of Na^+,K^+-ATPase as a cause of cellular energy expenditure in muscle. While it would be invalid to conclude that the role of Na^+,K^+-ATPase is of the identical magnitude in vivo as measured in vitro, the comparison of in vitro and in vivo estimates for rate of protein synthesis does provide some confidence that at least what is measured for these muscle preparations is indicative of what occurs in the animal.

In conclusion, activity of the Na^+,K^+-ATPase accounted for a minimum of 40% of muscle O_2 consumption. The energy

required for peptide bond synthesis was estimated to account for less than 4% of muscle O_2 consumption. The amount of muscle O_2 consumption due to activity of the Na^+,K^+-ATPase was similar for 7 month old calves of different breed backgrounds, and was increased for 10-21d old dairy calves. The amount of O_2 calculated to have been expended on peptide bond synthesis was increased 50% or greater for muscle from 10-21d dairy over that of older dairy calves, and from control DM over that of extreme DM calves. The increased amount of O_2 consumption expended in direct support of protein synthesis would not account for the difference in muscle O_2 consumption measured between the dairy groups.

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Table V.1 In vitro O_2 consumption, ouabain inhibition and Na^+,K^+-ATPase-dependent[1] and independent[2] respiration of calf muscle preparations.

Group	O_2 consumption (μl O_2 /mg/h)[3]	% Inhibition of O_2 consumption by ouabain	Na^+,K^+-ATPase-dependent O_2 consumption (μl O_2 /mg/h)[3]	Na^+,K^+-ATPase-independent O_2 consumption (μl O_2 /mg/h)[3]
10-21d dairy	3.27 ± 0.27**a**	41.3 ± 4.5**ab**	1.36 ± 0.11**a**	1.93 ± 0.16**a**
7 month dairy	2.75 ± 0.27**b**	39.4 ± 2.9**b**	1.08 ± 0.11**b**	1.67 ± 0.16**b**
control DM	2.44 ± 0.24**c**	42.7 ± 1.4**a**	1.04 ± 0.12**b**	1.40 ± 0.16**c**
extreme DM	2.61 ± 0.27**bc**	40.6 ± 2.2**ab**	1.06 ± 0.10**b**	1.55 ± 0.14**b**

[1] Na^+,K^+-ATPase-dependent respiration = total O_2 consumption x inhibition by ouabain.
[2] Na^+,K^+-ATPase-independent respiration = total O_2 consumption - Na^+,K^+-ATPase-dependent respiration.
[3] Values are expressed as mean ± S.E.
a,**b**,**c** Means within a column followed by different letters differ significantly ($p<0.05$).

Table V.2 Rate of ^{14}C-phenylalanine incorporation into protein of calf muscle preparations.

Group	Rate of ^{14}C-phenylalanine incorporation (*n*mole/mg/h)[1]	Ks[2] (%/d)	% O_2 consumption expended by protein synthesis[3]
10-21d dairy	0.023 ± 0.002a	1.5	3.3
7 month dairy	0.015 ± 0.002b	1.0	2.5
control DM	0.017 ± 0.002b	1.1	3.1
extreme DM	0.011 ± 0.001c	0.7	2.0

[1] Values expressed as mean ± S.E.

[2] Values for the fraction of muscle protein synthesized per day (Ks) were calculated assuming an average muscle amino acid molecular weight of 130 g/mole and a phenylalanine content of 3.0 mole % (Chang & Goldberg, 1978).

[3] The calculation of the fraction of muscle O_2 consumption expended for protein synthesis assumed 5 mole ATP to be required for incorporation of 1 mole of amino acid into a peptide chain and 1 mole O_2 to be required for synthesis of 5 mole ATP.

a,b,c Means within a column followed by different letters differ significantly ($P<0.05$).

Table V.3 Rate of tyrosine release from calf muscle preparations.

Group	*n*mole tyrosine/mg/h[1]
10-21d dairy	0.002 ± 0.009a
7 month dairy	0.002 ± 0.011a
control DM	0.016 ± 0.008b
extreme DM	0.078 ± 0.008c

[1] Values expressed as mean ± S.E.
a,b,c Means within a column followed by different letters differ significantly ($P<0.05$).

VI. O_2 CONSUMPTION AND Na^+, K^+-ATPase-DEPENDENT RESPIRATION IN MUSCLE OF LAMBS AND LACTATING AND NON-LACTATING EWES

A. Abstract

The in vitro rate of O_2 consumption and the portion of respiration inhibited by ouabain were determined for sternomandibularis preparations from lambs, their lactating dams, and non-pregnant, non-lactating (control) ewes. Measurements were repeated after a 5 week interval at which time lambs had been weaned for 2 weeks and their dams were no longer lactating (dry).

Muscle from lambs at 2 weeks of age had an O_2 consumption rate 25% greater ($p<0.001$) than at 7 weeks of age and 49% greater ($p<0.001$) than that of control ewes; at 7 weeks of age lamb muscle O_2 consumption was 21% greater ($p<0.001$) than that of control ewes. Muscle preparations from lactating ewes had an O_2 consumption rate 35% greater ($p<0.001$) than dry and control ewes. The O_2 consumption rate of muscle preparations from control ewes did not differ significantly between sampling periods.

10^{-5}M ouabain inhibited muscle O_2 consumption by 39%. Increased energy expenditure at the level of Na^+,K^+-ATPase accounted for 40% of the increased O_2 consumption rate of muscle from lambs of 2 weeks as contrasted to 7 weeks of age, and 60% of the increased O_2 consumption of muscle due to lactation.

B. Introduction

To understand the factors which affect maintenance energy requirements at the metabolic level, it is necessary to identify specific components of background or maintenance energy expenditure and to quantify their relative contribution under a variety of different physiological conditions.

The activity of Na^+,K^+-ATPase in counteracting the transmembrane movement of Na^+ and K^+ along their concentration gradients has been identified to be a major source of energy expenditure in the muscle of sheep and calves (Chapters IV & V). The extent to which physiological, genetic and environmental factors influence Na^+,K^+-ATPase activity has not been established, but up to 80% of the increased in vitro O_2 consumption for muscle from cold exposed sheep is due to increased energy expenditure at the level of the Na^+,K^+-ATPase (Chapter IV).

This study was undertaken to examine the effect of age and lactation on total in vitro muscle O_2 utilization and the proportion of respiration used in support of the activity of Na^+,K^+-ATPase.

C. Experimental

Muscle samples were obtained from four 2 week old nursing male lambs; their four lactating dams, 2-4 years of age; and four non-pregnant, non-lactating (control) ewes, 2-5 years of age. These sheep were all sampled 5 weeks later

after the lambs had been weaned for 2 weeks and their dams were no longer lactating. Animals were brought in from pasture 3-5 d prior to surgery and housed in a heated barn (approximately 20°C) where they were fed good quality grass hay free choice. Water was available at all times. Lactating and control ewes were fasted and nursing lambs separated from their dams overnight prior to surgery.

Measurement of respiration and Na^+, K^+-ATPase-dependent respiration

A section of the sternomandibularis muscle was taken from each animal and preparations were made following the method described in Chapter IV. Immediately upon removal from the animal, muscle sections were placed in cooled (15°C) HEPES buffer containing 10.0 mM glucose and 5.0 mM acetate as substrates. Two to three small tied muscle fiber bundles were prepared (20.0 x 0.5 mm) at room temperature with the aid of a dissecting microscope. Initial respiration rates were measured for each muscle fiber bundle in an O_2 electrode system (Chapter IV). The muscle preparations were incubated for 45 min in buffer containing 10^{-5}M ouabain. Respiration rates were again measured. Percent inhibition of respiration by ouabain was calculated using the ratio of the difference between initial and that after incubation to the initial respiration rate.

Statistical analysis

Rate of muscle O_2 consumption, portion of respiration inhibited by ouabain, and Na^+,K^+-ATPase-dependent and independent respiration were analysed using a one-way analysis of variance with groups as fixed effects.

D. Results

In the first period, muscle from lambs at 2 weeks of age had an O_2 consumption rate greater ($p<0.001$) than that of lactating and control ewes (Table VI.1). The rate of O_2 consumption for muscle from lactating ewes was 32% greater ($p<0.001$) than that of control ewes. Inhibition of respiration by ouabain ranged from 35.8 to 45.8% and was greatest ($p<0.001$) for muscle from lactating ewes.

In the second period, muscle from lambs at 7 weeks of age had an O_2 consumption rate greater ($p<0.001$) than that of dry and control ewes (Table VI.2). The O_2 consumption rates of muscle from dry and control ewes did not differ significantly. Inhibition of respiration by ouabain ranged from 35.2 to 41.7% and was lowest ($p<0.001$) for muscle from lambs at 7 weeks of age.

Na^+,K^+-ATPase-dependent respiration, the amount of O_2 calculated to have been expended by the muscle through Na^+,K^+-ATPase activity, was greater ($p<0.001$) for lambs at 2 weeks of age than lactating and control ewes (Table VI.2). Na^+,K^+-ATPase-dependent respiration for muscle from lactating ewes was 67% greater ($p<0.001$) than that of

control ewes. Na^+,K^+-ATPase-independent respiration, the residual portion of muscle O_2 consumption not accounted for by activity of the Na^+,K^+-ATPase, was greater ($p<0.001$) for muscle from lambs at 2 weeks of age than that of lactating and control ewes. Na^+,K^+-ATPase-independent respiration for muscle from lactating ewes was 13% greater ($p<0.005$) than that of control ewes.

Na^+,K^+-ATPase-dependent respiration did not differ significantly between lambs at 7 weeks of age and dry and control ewes. Na^+,K^+-ATPase-independent respiration was greater ($p<0.001$) for muscle from lambs at 7 weeks of age than from dry and control ewes. Muscle from dry and control ewes had similar values for Na^+,K^+-ATPase-independent respiration.

Comparisons of measurements during lactation and after weaning

Muscle from lambs at 2 weeks had an O_2 consumption rate 25% greater ($p<0.001$) than at 7 weeks of age. The proportion of respiration inhibited by ouabain was similar for lambs at both ages. Muscle from lambs at 2 weeks of age had a 28% greater ($p<0.001$) value for Na^+,K^+-ATPase-dependent respiration than at 7 weeks of age, and a 23% greater Na^+,K^+-ATPase-independent respiration than at 7 weeks of age. Muscle from one of the lambs differed from samples from the other lambs with a greater ($p<0.05$) proportion of respiration inhibited by ouabain at 2 and 7 weeks of age,

and a greater ($p<0.05$) muscle O_2 consumption rate at 7 weeks of age.

The O_2 consumption rate of muscle from lactating ewes was 35% greater ($p<0.001$) than when the ewes were dry. The proportion of respiration inhibited by ouabain was also greater ($p<0.05$) for muscle taken from ewes when lactating than when dry. Muscle from lactating ewes had Na^+,K^+-ATPase-dependent and independent respiration values 50% ($p<0.001$) and 24% greater ($p<0.001$), respectively, than when dry. Muscle O_2 consumption and the proportion of respiration inhibited by ouabain did not differ significantly between animals within groups in sampling periods.

The O_2 consumption rate of muscle from control ewes remained constant between sampling periods. Inhibition of muscle respiration by ouabain increased ($p<0.05$) from 36.8 to 39.8% for the second sampling period. As a result, Na^+,K^+-ATPase-dependent and independent respiration differed by 8% ($p<0.05$) for muscle from control ewes between sampling periods. Muscle O_2 consumption and the proportion of respiration inhibited by ouabain did not differ between animals within groups in sampling periods.

E. Discussion

Skeletal muscle is an important site of energy expenditure, estimated to account for 26-62% of the whole body O_2 consumption of dogs and small mammals (Martin & Fuhrman, 1955), and from measurements of cardiac output to body tissues (Christopherson et al. 1980), can be calculated to account for 37% of total cardiac output. Both the age related decrease in energy metabolism of sheep (Graham, 1967) and the increased maintenance energy requirements associated with lactation (Moe et al. 1972) were reflected in the in vitro O_2 consumption rates of muscle from lambs and ewes. The decrease in muscle O_2 consumption measured for lambs at 7 weeks as compared to 2 weeks of age, was the result of approximately equal proportional decreases in both the Na^+,K^+-ATPase-dependent and independent components of respiration. The effects of weaning and aging are not separated in the comparison of 7 and 2 week old lambs. The lower muscle O_2 consumption of dry and control ewes than of 7 week lambs was due to a decrease in Na^+,K^+-ATPase-independent rather than dependent respiration suggesting that either aging per se, or aging after 7 weeks influences muscle energy expenditure differently than aging and weaning.

Within 2 weeks of weaning, the O_2 consumption rate, portion of respiration inhibited by ouabain, and Na^+,K^+-ATPase-dependent and independent respiration of muscle from the ewes that had been lactating had decreased

to the values measured for muscle from control ewes. Lactating animals are known to have a greater feed intake than when dry; greater feed intake has been measured to cause increased O_2 consumption as a result of a greater amount of Na^+,K^+-ATPase-independent respiration in muscle from sheep (Chapter IV). Since they were allowed voluntary intake, it is likely that lactating ewes had a greater feed intake than dry and control ewes and this may have contributed to the greater muscle O_2 consumption of lactating than dry and control ewes. Nonetheless, of the increased muscle O_2 consumption measured for ewes when lactating, 60% was accounted for by increased energy expenditure at the level of Na^+,K^+-ATPase. Of relevance is the observation that cold exposure selectively increases Na^+,K^+-ATPase activity in the muscle of sheep (Chapter IV) and small mammals (Stevens & Kido, 1974; Guernsey & Stevens, 1977). The mechanism for cold-induced enhancement of enzyme activity may be at least partially achieved by increased enzyme synthesis as a result of enhanced thyroid activity (Chapter IV). Clearly, the physiological objectives of lactation are different from cold exposure, the former being to achieve the synthesis of milk and the latter to counteract the heat demand of the environment. However, as in cold exposure, it is conceivable that the very considerable endocrine changes (Hart et al. 1978) that occur during lactation may cause the enhancement of Na^+,K^+-ATPase activity.

Ouabain inhibited muscle O_2 consumption by an average of 39%, in agreement with values previously reported for muscle of sheep and cattle (Chapters IV & V). The magnitude of inhibition of respiration by ouabain is a function of the extent to which Na^+-K^+ transport does in fact account for O_2 consumption and also both ouabain accessibility to the enzyme and the equilibrium reached between formation and dissociation of the inhibitor-enzyme complex (Tobin & Brody, 1972). The exact proportion of Na^+,K^+-ATPase activity actually inhibited in our muscle preparations under the experimental conditions employed is not known. Therefore, our measurements of the proportion of muscle O_2 consumption required for support of Na^+,K^+-ATPase activity must be considered as minimum estimates. Underestimation of Na^+,K^+-ATPase activity resulting from incomplete inhibition of the enzyme would overestimate Na^+,K^+-ATPase-independent respiration. The metabolic processes which would account for the Na^+,K^+-ATPase-independent component of muscle O_2 consumption have not been identified. From estimates of the cost of protein synthesis in the energy metabolism of muscle from growing calves (Chapter V), the energy expended for peptide bond synthesis would appear to account for only a minor portion of Na^+,K^+-ATPase-independent respiration.

In conclusion, lactation was shown to increase O_2 consumption in muscle from ewes and to cause increased energy expenditure at the level of the Na^+,K^+-ATPase. The mechanism for increased Na^+,K^+-ATPase activity is not known.

Aging to 7 weeks plus weaning decreased muscle O_2 consumption and Na^+,K^+-ATPase activity in lambs. The amount of muscle O_2 consumption not explained by Na^+,K^+-ATPase activity is considerable and may be due to incomplete enzyme inhibition under the experimental conditions, or to as yet unindentified energy consuming metabolic processes.

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Table VI.1 O_2 consumption and proportion of respiration inhibited by ouabain for muscle from lambs and lactating and non-lactating ewes.

Group	Muscle O_2 consumption (μl O_2/mg/h)[1]	% Inhibition of respiration by ouabain
Period I		
2 week lambs	5.37 ± 0.33a	35.8 ± 2.3a
lactating ewes	4.75 ± 0.25b	45.8 ± 1.8b
control ewes	3.61 ± 0.20c	36.8 ± 3.2a
Period II		
7 week lambs	4.31 ± 0.29a	35.2 ± 3.0a
dry ewes	3.53 ± 0.20b	41.7 ± 3.9b
control ewes	3.56 ± 0.21b	39.8 ± 1.9b

[1] Values expressed as mean ± S.E.

a,b,c Means within a column and within a measurement period followed with different letters differ significantly ($p<0.05$).

Table VI.2 Na^+,K^+-ATPase-dependent[1] and independent[2] respiration for muscle from lambs and lactating and non-lactating ewes.

Group	Na^+,K^+-ATPase-dependent respiration ($\mu l\ O_2/mg/h$)[3]	Na^+,K^+-ATPase-independent respiration ($\mu l\ O_2/mg/h$)[3]
Period I		
2 week lambs	1.94 ± 0.19**a**	3.43 ± 0.22**a**
lactating ewes	2.19 ± 0.16**b**	2.56 ± 0.13**b**
control ewes	1.31 ± 0.12**c**	2.30 ± 0.21**c**
Period II		
7 week lambs	1.51 ± 0.16**a**	2.80 ± 0.23**a**
dry ewes	1.46 ± 0.15**a**	2.07 ± 0.19**b**
control ewes	1.43 ± 0.12**a**	2.13 ± 0.13**b**

[1] Na^+,K^+-ATPase-dependent respiration = total O_2 consumption × inhibition by ouabain.

[2] Na^+,K^+-ATPase-independent respiration = total O_2 consumption - Na^+,K^+ ATPase-dependent respiration.

[3] Values expressed as mean ± S.E.

a,b,c Means within a column followed by different letters differ significantly ($p<0.05$).

General Discussion and Conclusions

This study confirmed previous findings in work conducted with sliced tissue preparations that active transport of Na^+ and K^+, catalyzed by the plasma membrane Na^+,K^+-ATPase, is a major component of muscle energy expenditure. While sliced muscle exhibited a significant decrease in O_2 consumption as compared to intact muscle, inhibition of respiration by ouabain was not substantially altered. The low response to ouabain of O_2 consumption and heat production obtained in investigations conducted with intact organ preparations was shown to be due, at least in part, to prior inhibition of Na^+,K^+-ATPase by use of a buffer with a high content of $MgCl_2$. Na^+,K^+-ATPase activity accounted for 13-33% of mouse, and 29-46% of sheep and cattle skeletal muscle O_2 consumption. The lower ouabain inhibition of respiration for mouse muscle than ruminant muscle may be a function of the enzyme-inhibitor interaction rather than a real difference in the actual proportion of muscle O_2 consumption expended in support of active Na^+-K^+ transport. Species differences in enzyme succeptibility to inhibition by ouabain have been shown to exist. Na^+,K^+-ATPase from rats has been measured to require a greater concentration of ouabain for maximal inhibition than that from dogs, pigs, sheep and guinea pigs. The actual proportion of the total Na^+-K^+ transport inhibited by ouabain in the muscle preparations employed in this study is

not known, but may not have been complete if the enzyme was not fully accessible to ouabain.

The amount of energy expended for Na^+,K^+-ATPase activity was responsive to physiological and environmental factors. Long term cold exposure was found to result in specific stimulation of Na^+,K^+-ATPase activity in muscle from sheep. More rapid cycling of Na^+ and K^+ between the extra- and intra-cellular space would be a futile cycle and yield heat production. Cold exposure of mice resulted in an increased muscle O_2 consumption not due to specific enhancement of Na^+,K^+-ATPase activity. This may be a result of resistance of mouse Na^+,K^+-ATPase to inhibition by ouabain, or due to the presence of an alternate mechanism for cold-induced muscle thermogenesis. The mechanism of enhancement of Na^+,K^+-ATPase activity is not known, but was speculated to be due to either an increased amount of enzyme as a result of hormonal stimulation of protein synthesis, a changed enzyme efficiency, or a changed membrane permeability to Na^+ and K^+ resulting in increased substrate presentation to the enzyme.

Lactation was measured to result in greater muscle O_2 consumption compared to non-lactating ewes, due to a specific enhancement of Na^+,K^+-ATPase activity. Young lambs and calves had a greater rate of muscle O_2 consumption as contrasted to older animals. This was due to increases in both Na^+,K^+-ATPase-dependent and independent components of respiration.

In vitro estimates of protein synthesis for calf muscle preparations were in good agreement with measurements obtained in vivo by other workers. While the rate of protein synthesis was affected by age and breed, the amount of energy expended in support of peptide bond synthesis was a minor component of total energy expenditure by the sternomandibularis muscle.

Tyrosine release from muscle has been used by investigators as a measure of protein degradation. Significantly greater rates of tyrosine release were measured for muscle from control and extreme DM calves than for dairy calves, which may reflect differences in protein degradative capacity.